Final Report: Illinois-Indiana Sea Grant Development Project

Title of Project: Impacts of Nanomaterials on Aquatic Microbial Communities

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Abstract

The objective of the project was to assess the potential ecological impacts of nanomaterials (NM). Specifically, the project was designed to examine the effects of one widely used NM, nanotitanium dioxide (NTD), on microbial communities in aquatic ecosystems, specifically streams. The project was based on the hypothesis that NTD will exert a disruptive effect on benthic microbial communities by virtue of its nanoscale effects and it photoactive properties. We tested our hypothesis with a controlled manipulative experiment that examined the response of benthic microbial communities to exposure to NTD using model streams. Our results demonstrated significant effects of NTD on bacterial community size and denitrification rates. The pattern of the changes in community size and function suggested a shift in the sediment bacterial community composition from NTD sensitive organisms to NTD resistant organisms. This result is novel because there is no data in the literature that has demonstrated an effect of NTD on environmentally relevant bacterial species in natural or simulated natural environments. Ongoing molecular analysis (bacterial tag pyrosequencing) of the samples collected in this experiment will allow us to identify the members of the NTD and control bacterial communities down to the genus and in some cases species level, which will provide valuable insight into the effects of NTD on environmentally relevant bacterial species. Our work will make a valuable contribution to the literature on this important emerging contaminant.

Introduction

***Objectives**:*
The objective of the project was to assess the potential ecological impacts of nanomaterials (NM). Specifically, the project was designed to examine the effects of one widely used NM, nanotitania, on microbial communities in aquatic ecosystems, specifically streams. We chose to focus on streams because these are a likely route of entry of NM into the environment, and we chose to focus on microbial communities because 1) their activities are essential to the health of stream ecosystems, and 2) they can serve as convenient bioindicators of ecosystem responses to NM exposure.

Problem:
Nanomaterials (NM) are defined as materials with at least one dimension of 100 nm or less. The small size of NM gives them novel physicochemical properties. Specifically, due to the nano-scale dimensions of NM the surface area of nanoparticles is hundreds of times greater than that of the same mass of larger particles, making the NM more highly reactive. In addition, at the nano-scale the bonding among atoms is different (e.g. more stained) producing changes in optical, electrical, magnetic, thermal, mechanical and chemical properties.

NM are used in a diverse array of applications, including products ranging from pharmaceuticals and cosmetics to tools and electronics. In 2004 over 2,000 tons of engineered NM were produced, and the rate of production is expected to rise to greater than 50,000 tons per year over the next decade (Nowack and Bucheli 2007). Between 2004 and 2006 global investments in the nanotechnology (NT) industry approximately quadrupled to nearly $12 billion (Lux Research 2007) and forecasters predict global investments in NT to reach $27 billion by 2013 (McWilliams 2008).

Given the widespread commercialization of NM and the rapid growth of this industry, there is growing concern about the potential for unanticipated environmental consequences of these materials. Products produced for human consumption will inevitably be released into the environment at various points in their life cycle (production, use, disposal) and, over time, some of these products can accumulate and have negative environmental impacts (Robichaud et al. 2009). We have experienced this phenomenon previously with other commercial compounds (e.g. PCBs) and there is no reason to expect NMs to exhibit different fates. In addition, the very properties that make NM commercially useful (e.g. high reactivity, the ability to penetrate cells) suggest that they may have negative effects on biota (Borm and Berube 2008). Despite these concerns, the risks posed by nanotechnology to ecological and environmental health have not yet been rigorously assessed (Lubick 2008).

Rationale:
We chose to focus our research on nano titanium dioxide (NTD) for four main reasons: 1) it is one of the most widely used NM, 2) it has been demonstrated to have some negative effects on biota, 3) there were at the time no studies examining the effects of NTD on environmental microbial communities, and 3) one member of our research team

(PI Gray) had extensive prior experience with this material.

Titanium dioxide (TiO2) has long been in wide industrial and commercial use as a white pigment. The rutile crystal phase of TiO2, in the size range from 200-1000 nm, is considered environmentally and toxicologically benign and is commonly used in food, paint, and sunscreens. In contrast, NTD is transparent and has photocatalytic properties due to its ability to produce reactive oxygen species (ROS) when exposed to light. The commercial potential of NTD is massive, including applications in medicine, personal care products (sunscreens), architecture (paint, wall paper, tiles, etc.), the automotive and food industries (cleaner technologies, non-fogging glass and mirrors, product safety), and the textile and glass industries (Nanoposts 2008). The worldwide NTD market is currently over $2 billion annually and analysts predict continued growth.

The toxic effects of NTD are thought to be associated mainly with its ability to generate ROS under illumination. Exposure of living cells to ROS can damage DNA and other cellular components and, because of this, NTD has been used as an effective disinfectant on surfaces (Wolfrum et al. 2002). However, adverse biological effects of NTD have also been observed under dark conditions (Adams et al. 2006; Gurr et al. 2005). For example, Takeda et al (2009) noted that NTD administered to pregnant mice ultimately affected genital/cranial nerve systems in developing offspring.

There are some data in the literature that suggest that NTD has the potential to enter and persist in aquatic habitats. Recent research tracked the fate of NTD in a wastewater treatment plant and demonstrated that, while the majority of NTD adsorbed to biomass, 10-100 mg/L was discharged in the effluent (Kiser et al 2009). While there are few studies on the aqueous stability and aggregation of NTD under relevant field conditions (Christian et al, 2008), NTD is readily stabilized in aqueous solution by natural organic material, suggesting that environmental dispersion of NTD may occur to a far greater extent than originally anticipated (Hyung et al 2007; Domingos et al 2009).

There is very little data available on the effects of NTD on microbes within aquatic ecosystems. This represents a critical knowledge gap, as microbes within aquatic ecosystems play essential roles as drivers of biogeochemical cycles and as significant components of aquatic food webs.

Hypothesis:

This research project was based on the following hypothesis: nano titanium dioxide will exert a disruptive effect on benthic microbial communities by virtue of its nanoscale effects and it photoactive properties.

Narrative Report

Methods

We tested our hypothesis with a controlled manipulative experiment that examined the response of benthic microbial communities to exposure to NTD. The experiment was conducted in model streams housed within a 2,174 sq. ft artificial-stream laboratory

located in a greenhouse on the top floor of the Life Sciences Building at Loyola University Chicago (LUC). The laboratory contains forty-eight 4.35m recirculating fiberglass racetrack style streams (2 m x 0.5 m x 0.15 m) arranged in 8 banks of six streams. Water velocity in each stream is controlled via a paddlewheel powered by a Dayton DC Gearmotor (model 4Z129A; 1/8 HP) with Dayton Speed Controls (4Z827D). Paddlewheels for each bank of streams are connected to a common drive shaft. Streams receive attenuated natural light (50% reduction).

A set of six streams was lined with a mixture of coarse gravel, sand, and ground leaf litter as a carbon source, and filled with 60L dechlorinated tap water amended with inorganic nutrients (NO3 and PO4). Each model stream received 100g sediment collected from a local stream (Nippersink Creek) to provide an inoculum of microorganisms. Streams were run for 2 months to allow sediment microbial communities to become established.

After the two month pretreatment period three randomly selected streams were amended with 1mg/L Degussa P25 NTD. Degussa P25 was chosen as it is the most widely commercialized formulation of NTD. The remaining three streams were maintained as unamended controls.

For all six streams we collected composite sediment samples from each stream immediately prior to dosing (day 0) and at approximately weekly intervals post-dosing and performed the following analyses:

Bacterial numbers were determined based on heterotrophic plate counts using a standard method (Page, 1982) and direct epifluorescence counts using a modified standard method (Kepner Jr & Pratt, 1994). All counts were normalized based on grams of dry sediment.

Respiration was measured using a standard method (Hill et al., 2002). Briefly, 10 mL of sediment was placed into a black HDPE 50mL centrifuge tube (Cole-Parmer, Vernon Hills, IL) filled to the top (no head space) with well water. Water temperature and initial dissolved oxygen (DO) were measured using a YSI ProODO meter (YSI Inc. Yellow Springs, OH). Centrifuge tubes were capped, eliminating all air bubbles and incubated at room temperature (25°C) in the dark for 2 hrs, after which final DO was measured and respiration rates were calculated as mg O2 consumed time-1. Respiration rates were normalized by sediment surface area.

Denitrification rates were quantified using the chloramphenicol-amended acetylene (C2H2) inhibition technique (Knowles 1990; Royer et al. 2004).

DNA was extracted from sediment samples for analysis of bacterial community composition via tag pyrosequencing of 16S rRNA genes. Sediment samples for these analyses were stored at -80C, and DNA was isolated using the UltraClean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA). Successful DNA isolation was confirmed by agarose gel electrophoresis. For tag pyrosequencing of bacterial 16S rRNA genes

extracted DNA was sent to the Research and Testing Laboratory (RTL) (Lubbock, TX). PCR amplification was performed using primers 530F and 1100R (Boon et al., 2002). The 530F primer was chosen in order to obtain sequences for the V4 hypervariable region, which has been shown to provide species richness estimates comparable to those obtained with the nearly full-length 16S rRNA gene (Youssef et al., 2009). Sequencing reactions utilized a Roche 454 FLX instrument (Roche, Indianapolis, IN) with Titanium reagents.

Sediment and water samples were also collected for quantification of NTD and determination of size classes.

Results and Discussion

NTD impacted the size of the sediment bacterial communities as indicated by heterotrophic plate counts, but the impact changed over the course of the experiment (Fig. 1). Specifically, there was an initial decrease in bacterial numbers in the NTD

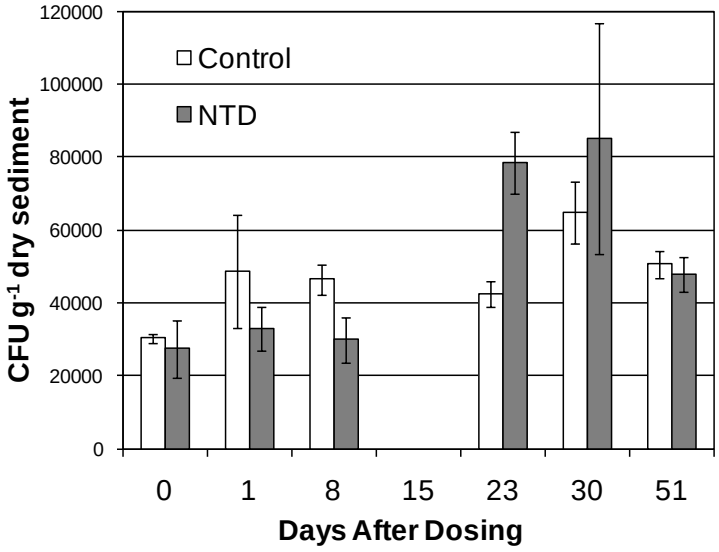

Figure 1. Numbers of bacteria in sediments of NTD treated and untreated control model streams measured over time based on heterotrophic plate counts.

treated streams as compared to the control streams, followed by an increase in bacterial numbers in the NTD treated streams as compared to the control streams, and finally the NTD treated streams returned to a bacterial community size that was equivalent to the control streams. One possible interpretation of these data would be that there was an initial die off of NTD sensitive bacteria, followed by a burst in the growth of NTD tolerant bacteria driven by the nutrients made available by the death of the sensitive organisms. Finally, once the nutrients from the dead bacteria were consumed, the bacterial community in the NTD streams returned to a community size

that was equivalent to the untreated control streams. If this interpretation is correct, it would be a very exciting finding as it would indicate that NTD had caused a shift in the composition of the sediment bacterial communities, with a decrease in the numbers of NTD sensitive organisms and an increase in the numbers of NTD tolerant organisms.

This result would be exciting as it would indicate a significant effect of NTD on sediment bacterial communities, something which has not been previously reported in the literature. This result would also make a significant contribution to scientific knowledge on this topic, as there is no data in the literature that suggests that different bacterial species differ significantly in their sensitivity to NTD. Indeed, most of the analyses that have been done to date have examined NTD effects only on *Escherichia coli*. Our work has the potential to provide powerful insights into the effects of NTD on environmentally relevant bacterial groups.

We also measured the activity of the sediment microbial communities in our streams, focusing on two specific processes, denitrification and respiration. We saw a significant short term effect of NTD treatment on denitrification rates (Fig 2). Specifically, at days 8 and 15 denitrification rates in the NTD treated streams were significantly higher than

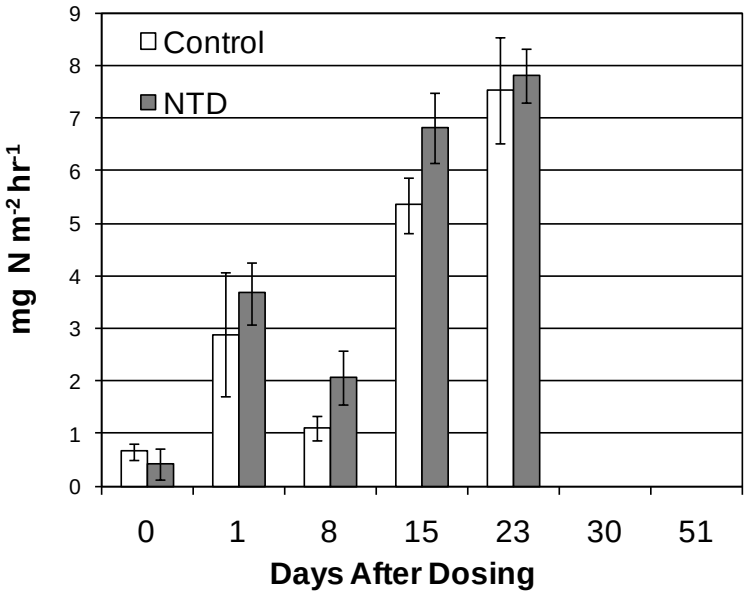

Figure 2. Sediment denitrification rates for NTD treated and untreated control model streams measured over time. Data for days 30 and 51 are still in progress.

denitrifcation rates in the untreated control streams. This pattern fits with the plate count data, and could be related to the death of NTD sensitive organisms and the subsequent growth of NTD resistant organisms due to the release of nutrients from the dead NTD sensitive cells. The denitrification results suggest that denitrification might have been a significant process in the breakdown of the dead cells.

We also measured respiration rates but observed no significant effect of NTD at any of the sampling dates. These results were surprising given the significant changes in bacterial community size that were observed (Fig. 1), but they suggest that respiration may not have been the dominant metabolic process involved in the decomposition of the dead NTD sensitive cells.

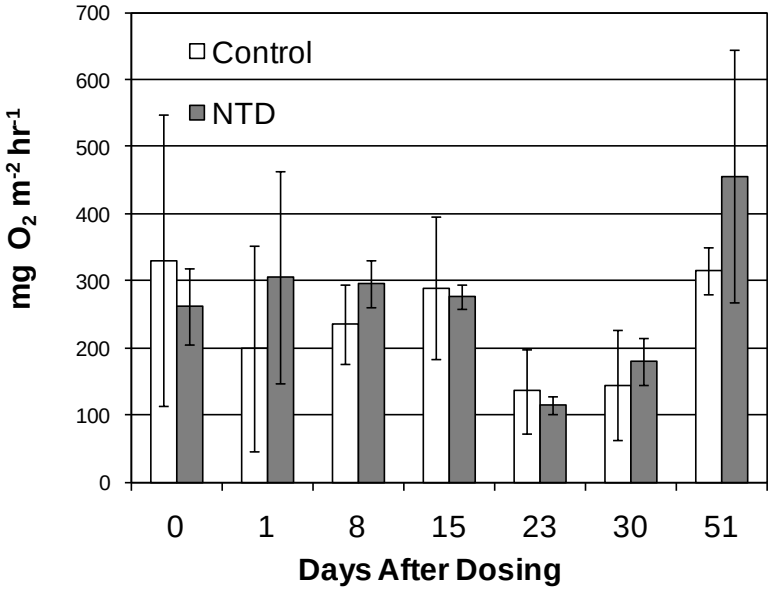

Figure 3. Sediment respiration rates for NTD treated and untreated control model streams measured over time.

Ongoing Analyses

We are currently analyzing bacterial community size in the samples from this experiment via a complementary technique, direct epifluorescnece cell counts. This work is in progress and should be completed shortly. It will be interesting to see if these estimates of overall bacterial community size follow the trends observed for the plate count assay, which targets only viable, fast growing aerobic heterotrophs.

We have also isolated DNA from all of the sediment samples and sent this DNA to RTL for tag pyrosequencing analysis of 16S rRNA genes. These data should be received with the next few weeks. Pyrosequencing data can be used to identify the members of bacterial communities down to the genus and in some cases species level. Analysis of these pyrosequencing data will provide powerful insights into the composition of the sediment bacterial communities and potential community shifts that have been caused by NTD exposure. As mentioned above, there is no data available in the literature on the possible effects of NTD on environmentally relevant bacterial groups, so these data will make a valuable contribution to the literature.

The varied responses of the bacterial communities to NTD over time that we observed (Figs. 1 and 2) may also reflect changes in the NTD distribution over the course of the experiment (e.g. precipitation, clumping). We collected water and sediment samples at each of our sampling times and the concentration and particle size of NTD in these samples are currently being analyzed in the Gray lab. These data should provide further insight into the fate of NTD in our model stream systems.

Keywords
Nanomaterials, Nanotitania, Sediment, Bacterial Communities

Partnerships
This IISG funded project was a partnership between PIs at LUC (Kelly and Peterson) and Northwestern University (Gray). The IISG grant enabled continuation of the ongoing partnership between these PIs, which had received prior NSF support and has now received new NSF support to extend the work of the IISG funded project.

Publications
We are planning to submit an abstract based on this work for a poster presentation at the 14th International Symposium on Microbial Ecology which will be held August 19-24, 2012 in Copenhagen, Denmark. The abstract submission deadline for this symposium is March 16, 2012.

We also plan to publish the results of this project in a peer reviewed journal such as *Environmental Science and Technology*, *Applied and Environmental Microbiology*, or *Freshwater Science*.

Undergraduate/Graduate Names and Degree
Although this development grant did not directly support any students, two students who received internal support from Loyola worked on this project. Specifically, one MS student in the LUC Department of Biology, Alexandra Ozaki, and one LUC undergraduate student, Erin Adams, worked on this project. Alexandra will be using this project as the basis for her MS thesis research.

Related Projects
During the period of this IISG development grant we received a new grant from the National Science Foundation, Chemical, Bioengineering, Environmental, and Transport Systems cluster. The title of the proposal is *The unintended ecological consequences of nanomaterials: effects of nanotitania in benthic systems.* We received $600,000 for a three year project. While this project is closely related to the IISG project in that both are focused on the ecological effects of NTD, the experiments included in the NSF project will be distinct. Specifically, the NSF project will focus on cellular toxicity of different NTD formulations via a high-throughput screening approach, and it will focus on effects of NTD on biofilm communities using small scale microcosms. We believe that this NSF award will help expand the impact of the IISG project by expanding our research efforts on this extremely important emerging contaminant, nanotitania dioxide.

Awards and Honors
NA

Patents/Licenses
NA.

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